COMPARATIVE ANALYSIS OF GUT MICROBES FROM ALZHEIMER'S MICE MODEL TREATED WITH *Bacopa monnieri* EXTRACT

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE
IN
MICROBIOLOGY

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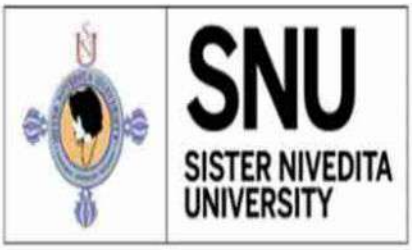

School of Health Science and Translational Research Sister Nivedita University
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CERTIFICATE

This is to certify that the M.Sc. dissertation thesis entitled **'Comparative analysis of gut microbes from Alzheimer's disease mice model treated with *Bacopa monnieri* extract'** is submitted by **Ms. Angela Sara Jacob**, Registration ID:230010040973 & Enrollment ID: 2322211001004 of M.Sc. 4th Semester of the Department of Microbiology/Biotechnology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata. The work leading to this thesis was done by her during the period, January, 2025 to May 2025, for the partial fulfillment of her Master's degree in Microbiology. She has worked independently under my guidance and completed it to my satisfaction.

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DECLARATION BY THE STUDENT

I hereby declare that the work which is being presented in this dissertation entitled " COMPARATIVE ANALYSIS OF GUT MICROBES IN Alzheimer's RICE MODEL TREATED WITH Bacopa monneiri EXTRACT " in partial fulfilment of the requirements for the award of the degree of Masters of Science in Microbiology is an authentic record of my own work carried out during the period from January 2025 to June 2025 under the supervision of Dr Mausumi Sikdar (ne'e Bhakta) Department of Life Sciences Presidency University College Street Kolkata -73 .

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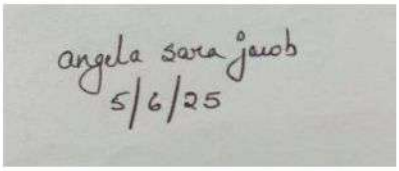

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TO WHOM IT MAY CONCERN

This is to certify that the project report titled **"Comparative analysis of gut microbes from Alzheimer's mice model treated with Bacopa monnieri extract"**, submitted by Ms. Angela Sara Jacob (Registration No.230010040973 .Enrolment No 2322211001004) to the Department of Microbiology, School of Health Science and Translational Research at Sister Nivedita University, Kolkata, is a bona fide record of research conducted by her for the partial fulfillment of the requirements for the Master of Science degree.

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ABSTRACT

Alzheimer's complaint(declaration) is a dynamic neurodegenerative complaint characterized by cognitive decay and memory disability. Later considerations have pushed the critical portion of the intestine- brain pivot in the pathogenesis of declaration, proposing that contrasts in intestine microbiota may affect neurodegenerative forms. Bacopa monnieri(BM), by and large known as Brahmi, is a conventional Ayurvedic condiment extremely popular for its neuroprotective and cognitive- improving bundles. This think about examines the modulatory merchandise of BM selection on intestine microbiota composition in a sodium azide- persuaded declaration mouse model.Eighteen womanish Swiss pale skinned person mice were partitioned into four bunches Control, Brahmi-treated, BM Azide-treated, and Azide- as it were. BM portion was arranged by drying Brahmi takes off at room temperature for 21 days, performing in a fine greasepaint(unique weight 7.5 g; last weight 5 g). The greasepaint was broken down in outright liquor(17 rate) and cleared out unperturbed for 48 hours. The selection was concentrated and managed orally at a measurement of 40 mg/ kg body weight for 21 progressive days. Sodium azide was managed intraperitoneally at 6 mg/ kg of body weight for 20 days to actuate declaration- suchlike side effects post-treatment, fecal tests were collected for microbiota investigation, and intestinal Akins were assembled for histological examination. Biochemical tests-urease, citrate utilization, methyl red-vogues- Proskauer(MR- VP), triple sugar press(TSI), coagulase, catalase, indole, DPPH radical scavenging, siderophore test, and Kirby- Bauer are a part of biochemical tests, were conducted to evaluate microbial movement and antioxidant potential.Histological examination uncovered that segments of the digestive system from the BM- treated gather displayed diminished aggravation and spared mucosal judgment compared to the Azide-only gather, which appeared noteworthy mucosal decay and rebellious penetration. Biochemical measures demonstrate improved antioxidant effort and diminished pathogenic microbial stack in the BM- treated groups.These discoveries recommend that BM portion applies a defensive impact on intestine microbiota composition and intestinal wellbeing in a declaration mouse demonstrate, possibly moderating neurodegenerative forms through the intestine- brain pivot.

Keywords:-gut micriobe, alzhiemer's,bacopa monneiri,femaleswiss albino mice, radial maze

KEYWORDS

Gut microbes, Alzheimer's disease, Female swiss mice, *Bacopa monneri,* Radial maze

INTRODUCTION

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative disorders worldwide, characterized by a gradual decline in memory, reasoning, and cognitive ability (Lane et al., 2018). With more than 55 million individuals currently affected globally, AD continues to place a significant burden on families, caregivers, and healthcare systems (Prince et al., 2015). The classical pathological features of AD include extracellular deposition of amyloid-beta (Aβ) plaques, intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein, mitochondrial dysfunction, and chronic neuroinflammation (De Strooper& Karran, 2016; Selkoe& Hardy, 2016).

Traditionally, AD research has focused on brain-specific mechanisms, but recent discoveries have expanded this perspective to include peripheral systems such as the gut. The gut-brain axis, a bidirectional communication network between the central nervous system and the enteric microbiota, is now known to influence neurodevelopment, cognition, behavior, and neuropathology (Cryan et al., 2019). Microbial metabolites like short-chain fatty acids (SCFAs), tryptophan derivatives, and lipopolysaccharides (LPS) can directly or indirectly impact neuroinflammatory pathways, blood–brain barrier integrity, and synaptic signaling (Shabbir et al., 2021).

Increasing evidence indicates that gut dysbiosis, or the imbalance in microbial diversity and abundance, is associated with the progression of Alzheimer's disease (Zhuang et al., 2018). Studies have shown elevated levels of pro-inflammatory taxa like *Escherichia/Shigella* and decreased abundance of commensal bacteria like *Lactobacillus* and *Bifidobacterium* in AD patients and mouse models (Vogt et al., 2017; Li et al., 2019). The resulting inflammation and loss of microbial homeostasis are believed to contribute to neurodegeneration via the activation of peripheral immune responses and increased permeability of the blood-brain barrier (Liu et al., 2019).

To explore the pathogenesis of AD and test potential treatments, researchers often rely on animal models that mimic human neuropathology. Among them, the sodium azide (NaN_3)-induced mouse model has gained attention for its ability to replicate key features of AD, including memory impairment, mitochondrial dysfunction, and amyloid accumulation (Kumar et al., 2017).Sodium

azide is a potent asset of cytochrome c oxidase(complex IV of the mitochondrial respiratory chain), and its administration causes oxidative stress, ATP reduction, and neuronal apoptosis(Yadav et al., 2019). (Yadav et al., 2019).

In mice, prolonged exposure to NaN_3 at low doses (commonly 6 mg/kg/day for 20 days) results in spatial memory deficits and behavioral changes resembling early AD (Patel et al., 2021). This model also induces histological changes in the brain and gut, making it suitable for studying systemic effects and the gut-brain axis in Alzheimer's disease.

In parallel to synthetic drugs, plant-based compounds have garnered increasing attention for their potential to combat neurodegeneration. Among these, *Bacopa monnieri* (commonly known as Brahmi), a traditional Ayurvedic herb, has long been used in India to enhance memory and learning (Pase et al., 2012). The primary bioactive components of *Bacopa monnieri* are bacosides A and B, which are known to cross the blood-brain barrier and exert multiple therapeutic effects, including antioxidant activity, inhibition of Aβ aggregation, and modulation of neurotransmitter levels (Aguiar & Borowski, 2013; Rauf et al., 2018).

Preclinical studies have shown that Bacopa improves synaptic plasticity, reduces lipid peroxidation, and upregulates the expression of brain-derived neurotrophic factor (BDNF) in hippocampal neurons (Uabundit et al., 2010). In AD models, Bacopa monnieri has demonstrated cognitive protection by restoring mitochondrial membrane potential and reducing neuroinflammation (Gohil et al., 2022). Furthermore, Bacopa has shown promise in regulating gut microbial populations, hinting at a possible dual mechanism of action through both the brain and gut (Banerjee et al., 2020).

The gut microbiome is a vast and dynamic ecosystem that plays a critical role in shaping immune and neuronal responses. Any disturbance in this microbial ecosystem can lead to systemic inflammation, which in turn exacerbates AD pathology (Kowalski &Mulak, 2019). Restoring gut microbial balance through prebiotics, probiotics, and phytochemicals is therefore a promising area of investigation in neurodegenerative diseases.

Herbs like Bacopa may exert their neuroprotective action partly by altering the gut microbiota. In animal models, *Bacopa monnieri* supplementation has been associated with increased abundance of beneficial bacteria such as *Lactobacillus plantarum* and reduced levels of LPS-producing bacteria (Sharma et al., 2022). These changes may lead to lowered gut inflammation and improved intestinal barrier function, which in turn reduces peripheral inflammation and neurodegeneration.

Despite increasing interest in the gut-brain axis, few studies have directly explored the combined effect of *Bacopa monnieri* on gut microbiota and cognitive function in a sodium azide-induced AD model. Given India's rich traditional medicine heritage and the urgent need for non-toxic, cost-effective neurotherapeutics, this study aims to fill a significant gap. It investigates whether Bacopa treatment can ameliorate behavioral deficits and gut dysbiosis in AD mice — and whether those changes can be quantified through behavioral tests, microbial assays, and histological gut

REVIEW OF LITERATURE

Alzheimer's disease (AD) is increasingly linked to gut–brain axis dysfunction: neurodegeneration coincides with gut dysbiosis and intestinal pathology *(Zhange et al)*. Animal studies show that AD-model mice exhibit altered gut microbiota composition and reduced levels of short-chain fatty acids (SCFAs)*(Chandra et al.)*. This may exacerbate cognitive decline via increased gut permeability and

systemic inflammation. Traditional Ayurvedic medicine uses *Bacopa monnieri* (brahmi), a perennial herb rich in bacoside saponins, for cognitive enhancement (Walker et al) . *Bacopa* extracts have potent neuroprotective, antioxidant, and metal-chelating effects, restoring memory and neuronal markers in toxin-induced amnesia models(Zhang et al.). Recent research suggests *Bacopa* might also modulate gut microbiota, providing prebiotic substrates that foster beneficial taxa.

Bacopa monnieri (brahmi) is a creeping aquatic herb (A: whole plant; B: stem; C: flower) prized in Ayurveda for memory enhancement (*Mathur et al.*). Its leaves contain bioactive triterpenoid saponins (Bacoside A/B) and flavonoids believed to underlie its nootropic action(Mathur et al).Classical texts describe Brahmi as a medhyarasayana ("intellect rejuvenator") that improves learning and recall. Modern studies confirm these claims: Bacopa extracts increase BDNF levels and upregulate hippocampal signaling (Akt/ERK pathways(*Mallick, Debasmit* et al)(*Mathur et al*). For example, one study reported that a 1-week *Bacopa* course significantly enhanced spatial working memory and hippocampal neurogenesis in young mice(*Pham et al)*. *Bacopa* restored learning by normalizing NMDA receptor subunit expression and cholinergic function (Mathur et al.) in toxin-induced amnesia. These cognitive gains are supported by antioxidant effects: *Bacopa* reduces oxidative stress markers and chelates neurotoxic metals, protecting synapses from amyloid-β damage. Taken together, *Bacopa monnieri* is neuroprotective and enhances cognition in rodent models, suggesting potential benefits for AD-related dementia.

Comparative behavioral testing in AD-model mice reveals both deficits and *Bacopa*-mediated improvements. Rats colonized with AD microbiota show significant cognitive impairments: decreased exploration, poor novel-object discrimination, and delayed escape latencies. In parallel, rodent AD models (e.g., sodium-azide or transgenic) display deficits on standard tests of learning and memory. Open Field Test (OFT) – measures general locomotor activity and anxiety. AD-like models often show hypoactivity or thigmotaxis (perimeter running)(Mathur et al.). *Bacopa*-treated mice, conversely, exhibit normalized exploration and increased center-crossings in OFT, reflecting reduced anxiety-like behavior. In one study, chronic stress decreased OFT locomotion, which *Bacopa* (80–120 mg/kg) fully rescued while also restoring hippocampal BDNF levels. Novel Object Recognition (NOR) – assesses recognition memory by quantifying time with a novel vs. familiar object. AD-model mice (or AD-FMT rats) spend less time exploring novel items, indicating memory loss (Grabucker, Stefanie et al). While direct AD/NOR studies with *Bacopa* are limited, improvements in analogous tests have been reported. For instance, *Bacopa* significantly increased exploration of novel locations/objects in scopolamine-amnesic mice, reversing memory impairment(*Mathur et al)*. Radial Arm Maze (RAM) The tests spatial working/reference memory. Performance is scored by errors (re-entering arms) or time to retrieve bait. In scopolamine-treated mice, *Bacopa* (CDRI-08 extract) markedly improved RAM performance: acquisition curves rose toward control levels, with fewer working/reference memory errors. This demonstrates *Bacopa*'s capacity to restore hippocampal-dependent spatial memory after toxin insult.AD mice also underperform on the Morris Water Maze (spatial learning) and Novel Location Recognition. *Bacopa* treatment generally mitigates these deficits. In an AD-transplant rat model, deficits in spatial tasks (water maze) and recognition were noted, indicating hippocampal involvement(*Granbucker,Stefanie et al*). *Bacopa*'s improvements are consistent across models: it normalizes escape latencies and increases novel-object preference in various paradigms. In summary, *Bacopa monnieri* enhances behavior in AD-like animals. It reverses memory impairments across tests, likely via synaptic repair and neurotransmitter modulation. Notably, studies by Indian groups (*Banerjee et al.*) have confirmed

these effects: *Bacopa* extract restored open-field locomotion and maze performance in stressed rats. These behavioral outcomes provide a functional readout linking *Bacopa*'s neuroprotective action to improved cognition.

Emerging evidence shows that AD-associated gut dysbiosis is accompanied by intestinal tissue changes. In the AD-FMT rat model, histology revealed clear pathology in the ileum and colon. Despite unchanged villus height, AD-colonized rats had significant goblet cell loss in the ileum (mucin-depleted epithelium)*(Granbucker,Stefanie et al*). This suggests thinning of the protective mucus layer, potentially allowing luminal antigens to penetrate. In the proximal colon, rats showed reduced organ length and an increase in crypt depth (hyperplasia/inflammation). Crucially, goblet cells were also depleted in the colon of AD microbiota recipients. These changes correlated with increased fecal water content, consistent with barrier dysfunction.

Such intestinal alterations are indicative of a "leaky gut" and local inflammation in AD-like states. Goblet cell loss and deeper crypts reflect mucosal injury, which could promote systemic inflammation and neuroinflammation. No studies to date directly report on *Bacopa*'s effect on gut histology in AD models; however, its known anti-inflammatory and antioxidant properties may protect gut integrity. In analogous contexts, botanical supplements have preserved mucosal structure. Taken together, AD gut dysbiosis is linked to ileal and colonic epithelial damage *(Granbucker ,Stefanie et a*l), and interventions that improve cognition (like *Bacopa*) may also mitigate such gut pathology.

Alzheimer's and related dementia are characterized by distinct gut microbial shifts. Clinical studies report that AD patients have a significantly lower Firmicutes/Bacteroidetes (F/B) ratio than controls. Specifically, beneficial Firmicutes (e.g.,*Faecalibacterium*, *Lactobacillus*, *Bifidobacterium*) are depleted, while Bacteroidetes and Proteobacteria (often inflammatory) are enriched. For example, *Vogt et al. (2017)*A decrease in Firmicutes and Bifidobacterium, accompanied by an increase in Bacteroidetes, was observed in the stools of Alzheimer's disease patients. Likewise, Liu et al. (2019) documented a decline in Firmicutes along with a rise in Proteobacteria as the disease progressed.

Experimental AD mouse models mirror these trends: APP/PS1 and 5xFAD mice show perturbed microbiota and reduced fecal SCFAs (acetate, butyrate, propionate) compared to wild-type. Lower SCFAs imply fewer SCFA-producing taxa (e.g. *Clostridium* clusters IV/XIVa, *Eubacterium*), which play key roles in gut and brain health.

In terms of specific taxa, studies note declines in SCFA-generators like *Eubacterium rectale* and *Faecalibacterium prausnitzii* in AD models. Concurrently, pro-inflammatory genera such as *Escherichia/Shigella* may flourish. These compositional changes can disrupt gut–brain signaling. Conversely, *Bacopa monnieri* appears to reverse some dysbiosis patterns. Preclinical evidence suggests *Bacopa* or its constituents enrich beneficial microbes: for instance, Bacopaside I (a *Bacopa* saponin) increased *Lactobacillus* and *Streptococcus* in depressed mice, linked to higher acetic acid levels. Another study noted that a multi-herb supplement containing *Bacopa* raised predicted butyrate producers in the gut microbiome. Although direct AD-mice data are limited, these findings imply *Bacopa* may boost SCFA-producers and elevate the F/B ratio. In sum, gut dysbiosis in AD involves a shift toward fewer Firmicutes and SCFA-producers, and *Bacopa* treatment may partially normalize this balance through its prebiotic-like effects.

Studies consistently show *Bacopa monnieri* improves cognitive metrics in AD models. In toxin-induced AD analogs (e.g., colchicine or amyloid injections), *Bacopa* ameliorated learning/memory deficits and reduced brain Aβ deposition via its anti-inflammatory/antioxidant action*(Mathur et a*l).

It also enhanced acetylcholine levels and reduced oxidative enzymes in hippocampus. Collectively, *Bacopa*'s cognitive benefits are well-documented through rodent behavior tests and molecular markers.

Emerging work links these brain effects to gut interactions. *Bacopa*'s phytochemicals may modulate the gut-brain axis by altering microbiota composition and reducing gut inflammation. In one depressed-mouse model, *Bacopa* extract increased abundance of beneficial genera (as above) and elevated fecal acetate(*Wang Jie et al)*. Another human trial noted that *Bacopa*-containing supplements can shift microbial pathways toward butyrate production. By fostering SCFA producers, *Bacopa* could improve gut barrier function and dampen peripheral inflammation, indirectly benefiting cognition. Furthermore, *Bacopa* exhibits direct antimicrobial effects: its extracts inhibit pathogenic gut bacteria (Kirby–Bauer assays show clear inhibition zones against *E. coli*, *Staphylococcus* spp.), and its antioxidants (DPPH assay) scavenge free radicals.

Several laboratories in India have substantiated *Bacopa*'s cognitive and gut benefits. For instance, *Banerjee et al. (2014)* demonstrated that *Bacopa* extract reversed chronic-stress-induced depressive behavior and restored hippocampal BDNF in female rats. Other Indian groups reported that *Bacopa* mitigates cognitive deficits from various neurotoxins (e.g., colchicine) by modulating cholinergic enzymes and antioxidant defenses (*Mathur et al*). While specific gut-microbe analyses in Indian AD models are scarce, these studies highlight *Bacopa*'s dual impact on brain function and systemic oxidative stress.

Bacopa monnieri exerts broad neuroprotective effects that extend to the gut. By enhancing SCFA production, reducing gut inflammation and oxidative stress, and supporting blood–brain barrier integrity, *Bacopa* may help break the vicious cycle of gut-derived inflammation in AD. Future work should directly compare the gut microbiota of AD-model mice with and without *Bacopa* treatment to elucidate these connections fully

Gut bacteria isolated from the ileum and colon can be profiled using classical microbiological assays. For example, Gram staining and colony morphology indicate basic identity. Biochemical tests—catalase, oxidase, coagulase, indole, methyl red, Voges–Proskauer, citrate utilization, urease, and triple sugar iron (TSI) agar—are then used to differentiate genera (e.g., *Escherichia* vs *Clostridium* vs *Lactobacillus*). Additionally, functional assays such as siderophore production (iron-chelation) and antioxidant capacity (DPPH radical scavenging) may be performed on isolates to assess probiotic potential. Antibiotic susceptibility is measured by Kirby–Bauer disk diffusion. These tests yield a "phenotypic fingerprint" of the gut microbiota. In comparative analysis, one would expect that *Bacopa*-treated AD mice will harbor different isolates or altered enzyme profiles (e.g., higher catalase or urease activity in beneficial species). While specific data on such biochemical tests in *Bacopa* studies are lacking, this approach allows linking microbial function to the observed shifts in gut health and host physiology

In recent years, research has begun to unravel how *Bacopa monnieri* may counteract gut dysbiosis in Alzheimer's models. Behavioral assays (OFT, NORT, radial maze) consistently show that *Bacopa* rescues cognitive impairments. Histological studies reveal that AD-associated microbiota damages the ileal/colonic mucosa (goblet cell loss, crypt hyperplasia), though the effect of *Bacopa* on such pathology remains to be explored. Metagenomic surveys indicate that AD involves a drop in SCFA-producers and a low Firmicutes/Bacteroidetes ratio *(Vogh et al)*, whereas *Bacopa* or its bacosides seem to enrich SCFA-generating genera (e.g., *Lactobacillus*, *Streptococcus*). Finally, standard bacterial assays (IMViC, enzyme tests, antibiotic sensitivity) can characterize the functional

implications of these shifts. Overall, *Bacopa monnieri* holds promises not only for cognitive enhancement but also for rebalancing gut microbiota and protecting intestinal health in Alzheimer's models. The collective findings from global and Indian studies underscore its dual role in brain and gut, warranting further investigations under the microbiota–gut–brain framework.

AIM: -To investigate the effect of *Bacopa monnieri* extract on the gut microbiome composition and activity in a Swiss albino mice model of Alzheimer's disease, and to compare microbial and behavioral changes across experimental groups.

OBJECTIVES: -

- To establish an Alzheimer's disease model in mice using sodium azide and validate it through behavioral assays.
- To administer *Bacopa monnieri* extract to both healthy and Alzheimer's-induced mice groups and observe its modulatory effect.
- To collect and culture fecal/gut samples from control, Alzheimer's-induced, treated, and combined-treatment groups for microbiological analysis

METHODS AND TREATMENT

2.1 Preparation of Bacopa monnieri Extract:-

Fresh Bacopa monnieri leaves were procured from a local market in Kolkata, India. The leaves were washed thoroughly and air-dried at room temperature (25°C) for 21 days to prevent degradation of phytoconstituents. The dried leaves were ground into a fine powder using a mechanical grinder. The initial weight of the leaves was 7.5 g, yielding 5 g of powdered material.

The powdered BM was subjected to maceration in absolute ethanol at a ratio of 1:7 (1 g powder in 7 ml ethanol) and left undisturbed for 48 hours at room temperature. The extract was then filtered using filter paper, and the filtrate was concentrated using a rotary evaporator at 40°C under reduced pressure to obtain a semi-solid extract. This process was repeated for seven consecutive days to ensure consistency and yield of bioactive compounds.

2 2Animal Grouping and Treatment Protocol:-

- Eighteen healthy Swiss albino mice (25–30 g) were acclimatized for one week under standard laboratory conditions with access to food and water. The mice were randomly divided into four groups:
- Control Group (n=1): Received food and distilled water for 21 days.
- BM Group (n=3): Administered BM extract orally at 40 mg/kg body weight daily for 21 days.
- BM + Azide Group (n=3): Administered BM extract as above and sodium azide intraperitoneally at 6 mg/kg body weight daily for 20 days.
- Azide Group (n=3): Administered sodium azide intraperitoneally at 6 mg/kg daily for 20 days.
- BM extract was diluted in distilled water at a ratio of 1:10 (1 mg extract in 10 µl water) to facilitate oral administration. Sodium azide was dissolved in distilled water at a concentration of 2 mg/ml for intraperitoneal injection

2.3.Delayed Spatial Win-shift (DSWS) Test on Radial Arm Maze Protocol:-

The test has two phases: Training (Sample phase) and Testing (Choice phase).In the sample phase, only a subset of arms (e.g., 4) is open and baited with food. The animal explores and collects food from these arms. After a delay period (e.g., 5 minutes to a few hours), the animal is placed back in the maze for the choice phase, where all arms are open, but only the previously unvisited arms are baited. The animal must remember which arms it has already visited and now explore the new, baited ones

2.4 Materials and Reagents:-

Cleaning Supplies:-

Paper towels

70% ethanol (v/v) diluted in distilled water for cleaning the maze between sessions.

Subjects:- Female Swiss Albino mice.

3.Housing Conditions:

Animals should be housed under normal controlled laboratory conditions in weight-matched pairs . Maintain a 12-hour dark/light cycle. Provide ad libitum access to food and tap water prior to the test phase. During testing, food access is restricted to ~80% of their normal intake to motivate food-seeking behavior. Record body weights at least three times a week to ensure that free-feeding weight does not drop below 80%.

4 Equipment

4.1. Radial Arm Maze (RAM):

For Mice: Use a smaller maze: central platform approximately 22 cm in diameter; arms around 25 cm long, 6 cm wide, and 6 cm high (transparent to allow visibility of external cues). Place the maze on the floor to avoid anxiety induced by elevation.

4.2. Visual Cues: Place a variety of salient and contrasting geometric shapes or colored objects on the walls surrounding the maze. These serve as extra-maze cues for spatial orientation.

4.3 Recording Equipment:- A digital video camcorder (phone model) mounted on a tripod or fixed overhead to capture the entire maze during each session.

5.Procedure: -

The procedure is divided into two main phases: The 2-day habituation phase and the experimental phase** (which consists of training and test sessions).

A. Habituation Phase (2 Days)

1. Goal: - Acclimate the rodents to the maze and its spatial cues.
2. Method Opening of Arms: All eight arms are left open.
3. Pellet Distribution: Place three standard chow pellets along each arm to stimulate exploration.

4. Session Details: Each animal is placed in the main area facing away from the investigator, ensuring a consistent introduction to the spatial cues. Conduct two 10-minute sessions per day with a 4hour interval between sessions.

5. PostHabituation: After the final session, return animals to their home cages. Provide approximately 20 pellets per animal.

6. Cleaning: Clean the maze with 70% ethanol after each animal and session, allowing it to dry completely before the next trial.

B. Experimental Phase: –This phase is conducted over multiple days (typically until the control group achieves a mean of ≤1 error in the test phase, often around 26 trials or 13 days).

1. **Session Structure (Daily Sessions)**:

- Each session is divided into a Training Phase, a 5minute InterTrial Interval (ITI), and a Test Phase: Sessions are separated by about 5 hours.

1.2 Training Phase (5 minutes):

- Arm Blocking: Only four of the eight arms are made accessible. The accessible arms are chosen pseudo-randomly with the condition that no more than two adjacent arms are open simultaneously (e.g., avoid combinations such as arms 1, 3, 4, 5 if three adjacent arms are accessible)

1.3 Baiting: - Bait each available arm's food well with three chow pellets.

- Behavior Monitoring: -Allow the rodent to explore for up to 5 minutes or until it retrieves rewards from all baited arms. Record any working memory errors (e.g., reentries into an arm already visited).

ii. Inter Trial Interval (ITI – 5 minutes):

Procedure:

- Return the animal to its home cage for 5 minutes.
- During this interval, clean the maze thoroughly with 70% ethanol, ensure arms are dried completely, and then open all eight arms.
- Bait the arms that were previously blocked (if applicable) for the test phase, while leaving the arms that had been accessible during training empty.

iii. Test Phase (5 minutes):

- Method: Place the rodent back in the maze with all arms open Allow the animal to explore the maze for 5 minutes.
- Behavior Monitoring:- Record both working memory errors (reentries into arms that were baited and already visited) and reference memory errors(entries into arms that were baited during the training phase but are now unabated).
- Recording: This phase can be video-recorded for later analysis using automated tracking software (FROM A PHONE) or manually scored.

2. **Continuation Criteria:** Continue the bi-daily sessions until the control group achieves a mean of no more than one error (across four trials binned as one data point) during the test phase.

3. **Additional Notes:**

Food intake is regulated to maintain (~80% of free-feeding body weight). Adjust food availability if the animal falls below this threshold. The training phase only indexes spatial working memory errors, whereas the test phase allows analysis of both working and reference memory errors.

4. **Data Analysis:-**

1. Error Calculation:- Calculate errors during the training phase (working memory errors) and during the test phase (both working and reference memory errors).

Binning: Combine errors from every four consecutive test trials and compute the mean value for each binning.

2. Statistical Approach Use a two-way analysis of variance (ANOVA) with "treatment" and "block" (trial bins) as factors .Follow up with Tukey's post-hoc tests as needed. Statistical significance is typically set at $p \leq 0.05$.

3. Other Measures: You may also analyze total errors, the number of correct entries before the first error, latency to the first arm entry, and the overall time to complete the test phase.

4. Troubleshooting and Considerations

1. If the Rodents Do Not Explore: Extend the habituation period or add extra sessions Increase the number of pellets during habituation to provide additional incentive.
2. Preventing Unwanted Behaviors: If a rodent attempts to jump between arms, gently remove it from the arm and return it to the center, facing away from you to minimize bias.
3. Consistency in Spatial Cues: Ensure that the extra-maze spatial cues (flags, shapes, colors) are visible and consistent throughout testing to allow reliable navigation.

3. Sample Collection: -

On day 22, mice were euthanized under anesthesia. Ileum and colon samples were collected aseptically for microbiota analysis. The entire gastrointestinal tract was excised, and sections of the small and large intestines were harvested for histological and biochemical analyses.

Isolation of single colonies from the sample

To isolate microbes from the gut of mice, Swiss albino female mice were brought to the Central Animal Facility at the Presidency Animal House.

They were kept in a quarantine room for seven days and were provided with a standard lab diet and unrestricted access to water.

After euthanasia, small intestinal sections (jejunum, ileum) were dissected out using sterile surgical instruments.

Homogenization was performed using a mechanical tissue homogenizer until a uniform slurry was achieved (usually 30–60 seconds).

The isolated gut was placed in a 1X PBS buffer to remove surface debris and blood clots. It was then transferred to a 1.5 ml Eppendorf tube containing Phosphate Buffer Saline (PBS) for homogenization using a micro pestle.
The pellet was discarded, and the supernatant was transferred to a tube containing a 1X PBS buffer.
. Serial dilutions were performed by transferring 100 µl of the supernatant PBS (pH~ 7.4) buffer to 900 µl of PBS buffer. This procedure was repeated to create dilutions from 10^-1 to 10^-3.
Selected dilution was spread on fresh Nutrient Agar plates and incubated overnight at 37◦c. 8. The plates with different dilutions were examined the following day and colonies of different morphologies were observed.
Selected colonies were streaked on fresh Nutrient Agar plates to obtain single colonies.

MATERIALS REQUIRED: -

- Distilled water
- Test tubes
- Glass plates
- Bacterial culture
- Eppendorf
- Surgical instrument
- PBS Buffer
- Nutrient agar
- Nutrient broth
- Muller-Hinton broth
- MHA plates
- Antibiotic disc
- Alcohol
- Crystal violet
- Test tube

Materials	Catalogue / NCBI number
Tips	Tarson
Microcentrifuge tube	Tarson
MM9	Merck
Muller Hinton Broth	HI media
Nutrient Agar	HI media
Nutrient Media	HI media

Muller Hinton Agar	HI media
Microplate Reader	Synergy Biotech Microplate Reader
DPPH ASSAY	Thermoscientific
Antibiotic Discs	HI media
Enterobacter	NCBI -PV3307010
S aureus	ATCC 25923

Muller Hinton Broth:-

Preparation of 50 ml of Muller Hinton Broth:-

Constituents	Standard value (g/l)	For media prepared (g/l)
Muller Hinton Broth	21	1.05
Distilled water	1000	300

1000 ml of distilled water needs 21g of the media.

Thus, 300 mL of distilled water needs 1.05g of media.

In a conical flask, the calculated amount of Mueller Hinton powder is added to distilled water

The mixture is stirred thoroughly

The flask is covered with a cotton plug and is sterilized in the autoclave at 121◦C at 15 psi for 15 minutes.

2. Nutrient Agar :-

Preparation of 300 ml of Nutrient Agar

Constituents	Standard value (g/l)	For media prepared (g/l)
Nutrient Broth	28	8.4
Distilled water	1000	300

1000 ml of distilled water needs 28g of agar

Thus, 300 mL of distilled water needs 8.4g of agar.

In a conical flask, the calculated amount of nutrient agar powder is added to distilled water

The mixture is stirred thoroughly

The flask is covered with a cotton plug and is sterilized in the autoclave at 121◦C at 15 psi for 15 minutes.

The sterilized agar is poured into a sterile petri dish in a sterile environment.

3. **Nutrient broth:-**

Preparation of nutrient broth

Constituents	Standard value (g/l)	For media prepared (g/l)
Nutrient Broth	13	1.3
Distilled water	1000	100

1000 ml of distilled water needs 13g of the media.
Thus, 100 ml of distilled water needs 1.3g of media.
In a conical flask, the calculated amount of Nutrient broth powder is added to distilled water
The mixture is stirred thoroughly
The flask is covered with a cotton plug and is sterilized in the autoclave at 121◦C at 15 psi for 15 minutes.

METHODS AND PREPARATION BIOCHEMICAL TESTS:

Gram Staining –
Principle: Gram staining is a differential staining technique used to classify bacteria into two main groups: Gram-positive and Gram-negative, based on differences in their cell wall structure. Gram-positive bacteria have a thick peptidoglycan layer that retains the crystal violet stain even after decolorization, appearing purple under the microscope. In contrast, Gram-negative bacteria have a thin peptidoglycan layer and an outer membrane containing lipopolysaccharides. This structure allows the crystal violet-iodine complex to be washed out with alcohol or acetone, after which the cells take up the counterstain safranin, appearing pink or red. The method, developed by Hans Christian Gram, helps in bacterial identification and antibiotic selection. This staining reaction is primarily based on how the bacterial cell wall interacts with the stains and reagents.

Gram Staining Protocol:
Materials Required:

- Clean glass slides

- Inoculating loop
- Bunsen burner or spirit lamp
- Crystal violet stain (primary stain)
- Gram's iodine solution (mordant)
- 95% ethanol or acetone (decolorizer)
- Safranin (counterstain)
- Distilled water.
-

Protocol:-

- Rinse with distilled water and blot dry using blotting paper.
  Observe the slide under oil immersion (100x objective Place a drop of sterile water on a clean slide.
- Using an inoculating loop, transfer a small amount of the bacterial colony and mix with the water to form a thin smear.
- Allow it to air dry completely
- Pass the dried slide quickly through a flame 2–3 times (smear side up) to fix the bacteria onto the slide.
- Flood the smear with crystal violet and leave it for 1 minute.
- Rinse gently with distilled water. Cover the smear with Gram's iodine solution for 1 minute.
- Rinse again with distilled water
- Tilt the slide and apply 95% ethanol dropwise until the runoff is colorless (usually 10–15 seconds).
- Immediately rinse with distilled water to stop decolorization
- Apply safranin to the smear and leave it for 30–60 seconds. Gram-positive cells will appear purple, and Gram-negative cells will appear pink/red.

BIOCHEMICAL TESTS:-

1. Indole test

The indole test is a biochemical test performed on bacterial species to detect the ability of an organism to degrade the amino acid tryptophan and produce indole. The test is based on the presence of an enzyme system called "tryptophanase" that converts tryptophan into indole.

The indole test is used to determine the ability of an organism to produce indoles from tryptophan.

Preparation of tryptone broth

Preparation of 40 ml of tryptone broth

Constituents	Standard value g/l	For experiment g/l
Tryptone	10	0.4
Sodium chloride	5	0.2
Distilled water	1000	40

Procedure:-

Inoculation - Using a sterile inoculating loop, a small amount of the bacterial culture was picked and inoculated into a test tube containing tryptone broth.

Incubation - Incubate the inoculated broth at 35-37°C for 24-48 hours. Ensure the culture is aerated during this period.

Addition of Kovac's Reagent - After incubation, about 0.5 mL of Kovac's reagent was added to the broth. Kovac's reagent contains p-dimethylaminobenzaldehyde, which reacts with indole.

Observation - Gently swirl the tube to mix. A red or pink layer will form at the top of the broth if indole is present. If there is no color change, indole is not produced.

Interpretation - Positive Result: Red or pink ring at the top indicates the presence of indole. Negative Result: No color change or yellow ring indicates that indole is not produced.

This test is commonly used to differentiate between species, particularly among Enterobacteriaceae.

2. MR-VP Tests (Methyl Red & Voges-Proskauer)

Principle:- The MR VP test, or Methyl Red and Voges-Proskauer test, is a biochemical method used to differentiate enteric bacteria based on their pattern of glucose metabolism. It involves two separate reactions within the same glucose phosphate broth. In the Methyl Red (MR) test, the focus is on the ability of bacteria to perform mixed acid fermentation. Some bacteria ferment glucose and produce stable acidic end-products like lactic, acetic, and formic acid, significantly lowering the pH of the medium. When the methyl red indicator is added, a red color confirms acid production, indicating a positive result. On the other hand, the Voges-Proskauer (VP) test detects organisms that ferment glucose but quickly convert the acidic products into more neutral compounds, particularly acetoin and 2,3-butanediol. When Barritt's reagents (alpha-naphthol and potassium hydroxide) are added, the presence of acetoin reacts to produce a pink to red coloration after aeration, indicating a positive VP result. This test is particularly useful in distinguishing between organisms such as *Escherichia coli* (MR positive, VP negative) and *Enterobacter aerogenes* (MR negative, VP positive). Proper incubation time, pH maintenance, and fresh reagents are crucial to ensure reliable outcomes.

INGREDIENTS: -

Constituents	Standard value g/l	For experiment g/l
Dextrose	5	0.2
Dipotassium phosphate	5	0.2
Sodium chloride	1.4	0.056
Disodium phosphate	1.96	0.0784

Dihydrogen potassium phosphate	0.84	0.033
Peptone	2.8	0.112

Procedure:

Inoculation and Incubation (Common for Both MR and VP):

Aseptically inoculate MRVP broth (5–10 ml in each tube) with a loopful of the test organism.

Inoculate separate tubes for each control strain.

Incubate all tubes at 35–37°C for 48 hours. (Extended incubation up to 5 days)

This test is particularly useful for differentiating between enteric bacteria, such as *Escherichia coli* (positive) and *Enterobacter aerogenes* (negative).

2.2 Voges Proskauer test

Procedure: -

Inoculation- Using a sterile inoculating loop, a test tube containing MR-VP broth is inoculated with the bacterial culture.

Incubation:- The inoculated broth is kept in an incubator at 35-37°C for 48 hours to allow fermentation.

Addition of Barritt's Reagents:- After incubation, 15 drops of Barritt's Reagent A (alpha-naphthol)is added to the broth followed by the addition of 5 drops of Barritt's Reagent B (potassium hydroxide).

Mixing- the tube is gently swirled to mix the reagents with the broth.

Observation- the mixture is allowed to stand for about 15-30 minutes. A color change will indicate the presence of acetoin.

Interpretation- Positive Result: If the solution turns pink or red, it indicates that the organism is capable of producing acetoin.Negative Result: If the solution remains yellow or does not change color, acetoin is not produced.

The VP test is particularly useful for distinguishing between enteric bacteria, such as *Enterobacter aerogenes* (positive) and *Escherichia coli* (negative.

Test result	Methyl Red	Voges Proskauer
Positive	Red color	Brownish red to pink
Negative	Yellow color	Brownish green to yellow

3. Simon Citrate utilization test :-

The citrate utilization test is used to determine an organism's ability to use citrate as its sole carbon source for growth. This test is often used to differentiate among gram-negative bacteria, particularly within the Enterobacteriaceae family.

Preparation of simon citrate agar :-

Constituents	Standard value (g/l)	For experiment (g/l)
Ammonium dihydrogen phosphate	1	0.1
Magnesium sulfate	0.2	0.02
Dipotassium phosphate	1	0.1
Sodium citrate	2	0.2
Sodium chloride	5	0.5
Bromothymol blue	0.08	0.008
Bacteriological agar	15	1.5
Distilled water	1000	100

Procedure :-

Inoculation- Using a sterile inoculating loop, the surface of a Simmons citrate agar slant was streaked with the bacterial culture. Ensure even coverage but avoid excessive inoculation.

Incubation- The inoculated slant is incubated at 35-37°C for 24-48 hours.

Observation- After the incubation period, the slant for color change is observed.

Interpretation- Positive Result: If the slant turns from green to blue, it indicates that the organism has utilized citrate, leading to the production of alkaline byproducts that raise the pH.Negative Result: If the slant remains green, the organism is unable to utilize citrate as its sole carbon source.

The citrate utilization test is particularly useful for differentiating species such as *Escherichia coli* (negative) from *Klebsiella pneumoniae* (positive) and *Enterobacter aerogenes* (positive).

4. Urease test :-The urease test is used to determine an organism's ability to hydrolyze urea into ammonia and carbon dioxide, indicating the production of the enzyme urease. This test is particularly useful for identifying certain gram-negative bacteria.

Constituents	Standard value g/l	For experiment (75ml) g/l
Dextrose	7.5	0.075
peptone	7.5	0.075
Urea	20	1.5
Phenol red	0.012	0.0009
Potassium phosphate monobasic	2	0.15
Sodium chloride	5	0.375
Agar	15	1.125

Procedure

Inoculation - Using a sterile inoculating loop, a test tube containing urea agar or urea broth is inoculated with the bacterial culture. If using a slant, streak the surface evenly.
Incubation - the inoculated medium is incubated at 35-37°C for 24 hours.
Observation- After incubation, the color change in the medium is observed.
Interpretation- Positive Result if the medium turns pink, it indicates that urease is produced, leading to the hydrolysis of urea and an increase in pH due to ammonia production. Negative Result - If the medium remains yellow or unchanged, urease is not produced.
The urease test is particularly useful for differentiating species such as Proteus vulgaris (positive) and Escherichia coli (negative).

5. Triple Sugar Test: -

Principle: - The Triple Sugar Iron (TSI) test is used to differentiate Gram-negative enteric bacilli based on their ability to ferment sugars (glucose, lactose, and sucrose), produce gas, and generate hydrogen sulfide (H2S).
INGREDIENTS:-

Constituents	Standard value g/l
Peptone	20
Beef Extract	3
Yeast Extract	3
Glucose	1
Lactose	10
Sucrose	10
Ferrous sulfate	0.2
Sodium chloride	5
Sodium Thiosulfate	0.3
Phenol red	0.024
Agar	13

Distilled water	1000 ml

Procedure:-
Label the TSI agar slant tube .Using a sterile inoculating needle, pick a pure bacterial colony.
Stab the needle deep into the butt of the agar (almost to the bottom).
Streak the slant .
Incubate the tube at 37°C for 18–24 hours.
Observe and record the color of the slant and butt, presence of gas (bubbles/cracks), and black precipitate.

6. Catalase test

Principle:-The catalase test is a biochemical test used to identify organisms that produce the enzyme catalase. This enzyme breaks down hydrogen peroxide (H_2O_2) into water and oxygen, which can be observed as bubbling or foaming when hydrogen peroxide is applied to a culture.

Procedure:
1. Culture Preparation: A pure culture of the organism to be evaluated is obtained.
2. Hydrogen Peroxide: A few drops of hydrogen peroxide is added to the slide.
3.Application: A small amount of the culture was placed on the hydrogen peroxide.
4. Observation: Looked for immediate bubbling or effervescence.

Interpretation - Positive Result: Presence of bubbles indicates that the organism produces catalase (e.g., Staphylococcus).Negative Result: No bubbles indicate a lack of catalase production (e.g., Streptococcus).
The catalase test helps differentiate between catalase-positive and catalase-negative bacteria, which is useful in identifying species and understanding their metabolic capabilities.

7. Coagulase test

Principle:-The coagulase test is a laboratory procedure used to differentiate Staphylococcus aureus from other staphylococci by detecting the presence of the coagulase enzyme, which causes blood plasma to clot.
Procedure:
1. Culture Preparation: A pure culture of the organisms to be tested is obtained.
2. Serum : A few drops of serum were taken on a fresh slide.
3. Inoculation: A small amount of bacterial culture was added to the serum
4. Observation: Checked for clot formation.
Interpretation- Positive Result: Clot formation indicates the presence of coagulase (e.g., Staphylococcus aureus).Negative Result: No clot formation indicates a lack of coagulase (e.g., Staphylococcus epidermidis).
Importance: The coagulase test is crucial for identifying pathogenic strains of Staphylococcus, particularly in clinical settings, to guide appropriate treatment.

8. Antibiotic susceptibility test :-

Principle: Antibiotic testing is essential for determining the effectiveness of antibiotics against specific bacteria. Here are the main methods used for antibiotic susceptibility testing:
Disk Diffusion Method (Kirby-Bauer Test)
Antibiotic-impregnated paper disks are placed on an agar plate inoculated with the bacteria. As the antibiotic diffuses, it creates a concentration gradient, and the area around the disk where bacteria cannot grow is called the inhibition zone.Antibiotic disc used - norfloxacin , vancomycin , ampicillin , amikacin , cephalexin.
Procedure:

- A bacterial lawn on an agar plate is prepared.
- Antibiotic disks are placed on the surface.
- The plate is incubated at 35-37°C for 24 hours
- Inhibition zones are measured after incubation.

Interpretation: The diameter of the inhibition zones is measured to classify bacteria as susceptible, intermediate, or resistant based on standardized guidelines.

ASSAYS:-

SIDEROPHORE ASSAY:-
Principle:-Siderophores are iron-chelating compounds secreted by microorganisms when iron is limited. The CAS (Chrome Azurol S) assay is commonly used to detect siderophore production. In the CAS dye, iron (Fe^{3+}), and hexadecyltrimethylammonium bromide (HDTMA) form a blue complex. When a siderophore is present, it removes Fe^{3+} from the CAS complex, causing a color change from blue to orange/yellow.

Ingredients:-

Constituents	Value for 500 ml
Sucrose	5 g
Potassium Dihydrogen Phosphate	0.5
Magnesium chloride	0.1g
Ammonium sulfate	0.5 g
Calcium chloride	5.54 g
Sodium chloride	2.5 g
Dipotassium phosphate	0.008 g

Material:-
Inoculated bacterial culture (grown in iron-deficient medium)

2 mL Eppendorf tubes

Centrifuge

96-well microtiter plate

CAS reagent (100 µL per well)

Shuttle solution (10 µL per well)

Micropipettes

Incubator (37°C)

Plate reader (or spectrophotometer) at 630 nm

Procedure:
Inoculation:
Inoculate the test bacteria in 2 mL iron-deficient medium and incubate for 48 hours at 37°C in a 2 mL Eppendorf tube.

Centrifugation:
After incubation, centrifuge the culture at 7000 rpm for 10 minutes to obtain the cell-free supernatant.

Sample Preparation:
Pipette 100 µL of the supernatant into each well of a 96-well microtiter plate.

Addition of Reagents:
Add 100 µL of CAS solution and 10 µL of shuttle solution to each well containing the sample.

Incubation:
Incubate the plate at 37°C for 1 hour to allow color change.

Measurement:
Measure absorbance at 630 nm using a microplate reader.

INTERPRETATION :-

OBSERVATION	INTREPRETATION
DECREASE IN RED COLOUR	POSITIVE
NO COLOUR CHANGE	NEGATIVE

DPPH ASSAY:-

Principle:-DPPH (purple) is reduced by antioxidants to DPPH-H (yellow). Measuring absorbance decrease at 517 nm reflects antioxidant activity.
INGREDIENTS

PROCEDURE:-

- Inoculate bacterial cultures in 10 mL BHI broth in a 2 microliterEppendorf
- Incubate at 37°C for 24 hours under shaking conditions.
- Centrifuge the broth at 8000 rpm for 10 minutes to collect cell-free supernatant.
- Pipette out 100 ml of the supernatant in a 96 well plate.
- Then pipette out 100 ml of dpph assay to it.
- Incubate in the dark at room temperature for 30 minutes.
- Take absorbance readings at 517 nm using a blank (methanol + DPPH only

Biofilm Development Assay: -

Isolated pure colonies were first inoculated into Tryptic Soy Broth for overnight inoculation from NA plates. Subsequently, the bacteria were transferred into a microplate into a TSB medium with additional 0.5% glucose supplementation. The plate was then kept at 37 ºC in an incubator for one week. After one week supernatant was pipetted out and 0.1% crystal violet (CV) was added. Then excess CV was pipetted out. To quantify the biofilm formation Each well was loaded with 30% Glacial acetic acid and mixed well. At 550nm spectrophotometric analysis was done to quantify the percentage of biofilm formation.

Tissue processing:-

1. Tissue Fixation:-

a. Choose the Appropriate Fixative Based on Tissue Type:
Buffered Formalin: Tissues: Liver, kidney, spleen, pancreas, heart, thyroid, lungs, adrenal
Duration: 48–72 hours
Bouin's Solution:-Tissues of Testis, ovary, uterus, epididymis, cerebrum, cerebellum
Duration: 6–24 hours

b. Initial Fixation and Refresh:
1. Place the tissue in the chosen fixative and allow it to remain untouched for the first 30 minutes.

2. After 30 minutes, perform a change of fixative. If working with larger specimens (such as kidney, spleen, pancreas, cerebrum, or cerebellum), it is advisable to cut the tissue into two halves or more pieces to ensure better fixative penetration.
3. Re-dip the tissue in fresh fixative, using a volume ratio of approximately 1:10 (tissue to fixative), and continue the fixation for the remaining duration.

2. Dehydration Process

Transition the tissue through a graded series of alcohols to gradually remove water:

Alcohol Concentration	Duration**
50% Alcohol	45 minutes
70% Alcohol	45 minutes
80% Alcohol	45 minutes
90% Alcohol	45 minutes
100% Alcohol	30 minutes (repeat twice)

Note: The last step with 100% alcohol is performed in two changes to ensure complete dehydration.

3. Clearing and Paraffin Infiltration: -

a. Clearing:
Xylene/Benzene Clearing:
- Immerse the tissue in Xylene or Benzene for 20 minutes per change.
- Make 3 separate changes to effectively remove the alcohol.

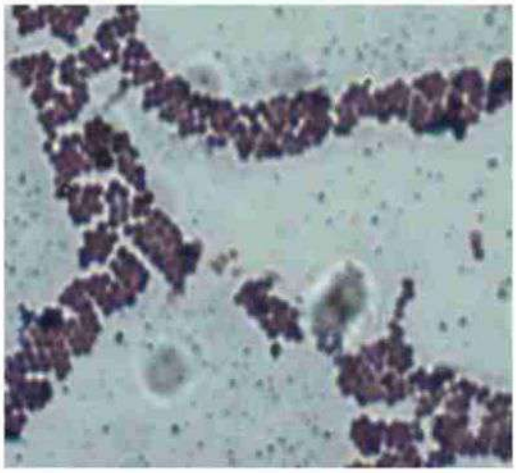
b. Transition to Paraffin:
Benzene (xylene)/Paraffin Mixture:
Immerse the tissue in a 1:1 mixture of Benzene (or xylene) and paraffin for 30 minutes.
This step is typically done overnight to ensure thorough replacement of the clearing agent.

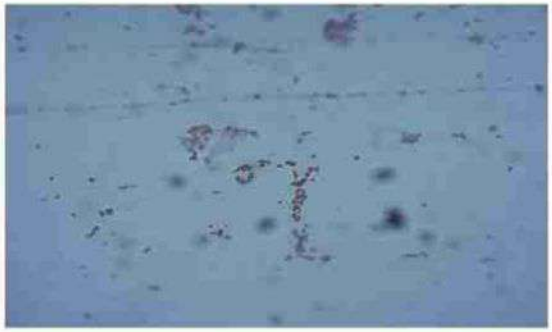
c. Paraffin Infiltration:

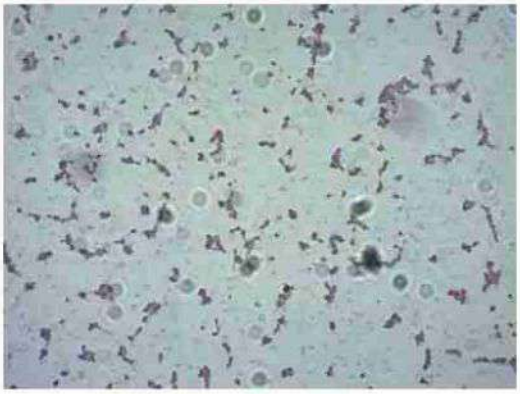
Melted Paraffin:
1. Immerse the tissue in pure melted paraffin (maintained at 50°–60°C) for 40 minutes.
2. Repeat this immersion for a second 40-minute cycle to secure complete infiltration.

4. Block Preparation:-
1. After paraffin infiltration, prepare the tissue block by embedding the specimen in paraffin.
2. Place the prepared block in water for 30 minutes (this may help in smoothing or final setting steps) and finally store the block at 4°C for long-term preservation.

RESULTS AND DISCUSSIONS: -







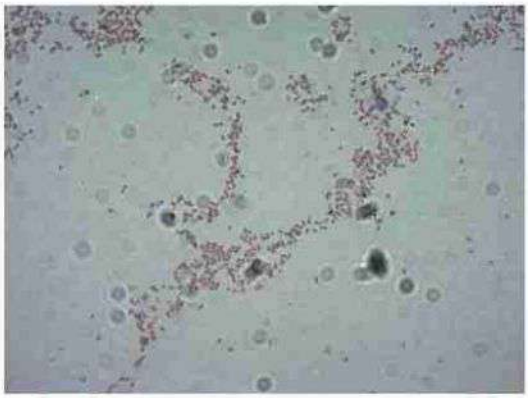


Figure 1,2,3,4:-Gram Staining.

Observation table for Gram staining

MAIN GROUP	MAIN SUBGROUP	COLOUR	Mixed STAINING	SHAPE
CONTROL	IILEUM	IILEUM 1	negative	coccus
AZIDE	COLON	COLON 2	positive	rods
AZIDE	COLON	COLON 1	positive	coccus
AZIDE+BM	COLON 2	COLON 2	positive	coccus

AZIDE +BM	COLON1	COLON 1	positive	coccus
	COLON 2	COLON 2	positive	rods
AZIDE +BM	ILEUM	ILEUM 1	positive	rods
AZIDE+BM	COLON	COLON 1	positive	coccus
BM	ILEUM	ILEUM 2	negative	coccus
BM	ILEUM	ILEUM 2	negative	coccus
Azide	Colon 2	colon 2.2	positive	coccus

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F

Figure 5 MIXED gram staining

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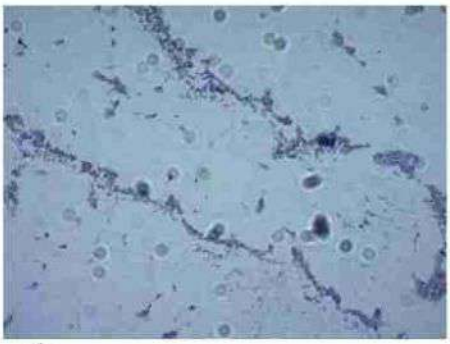

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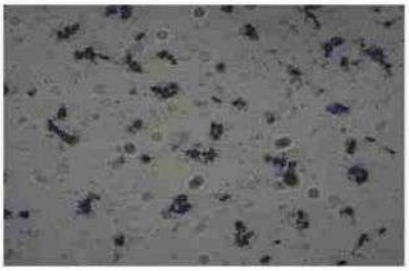

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MAIN GROUP	MAIN GROUP	Gram stain	Characteristics
AZIDE	COLON1	mixed	more gram-negative rods, less gram-positive coccus.
AZIDE	COLON 3	mixed	gram negative rods, gram positive coccus
AZIDE	ILEUM 1	mixed	gr negative rods, less positive bacteria shaped not defined.
AZIDE	ILEUM 2	mixed	gram negative rods, less gram-positive bacteria
AZIDE	COLON 1	mixed	gran negative rods, gram positive rods dark as well another small gram-negative rods
AZIDE + BM	ILEUM 2	mixed	Gram positive coccus, gram negative rods
AZIDE + BM	ILEUM 1	mixed	gram negative coccus and gram-positive rods
AZIDE + BM	COLON 1	mixed	gram positive coccus and gram-negative coccus
AZIDE + BM	ILEUM 1	mixed	gram positive coccus and gram-negative rod shaped
BM	COLON 1	mixed	Gram negative coccus , gram positive coccus
BM	COLON 2	mixed	Gram negative small rods, gram positive rods
BM	ILEUM 1	mixed	Gram negative as well as gram positive
BM	ILEUM 1	mixed	gr negative small rods, isolated gram
BM	COLON 1	mixed	more gram-negative strains
BM	ILEUM 1	mixed	gram negative coccus and less gram positive
BM	ILEUM 3	Mixed	gram positive coccus and gram-negative rods
BM	COLON 1	Mixed	gr Positive coccus, gr negative rods
BM	COLON 2	Mixed	rods shaped negative

IMViC TESTS:-

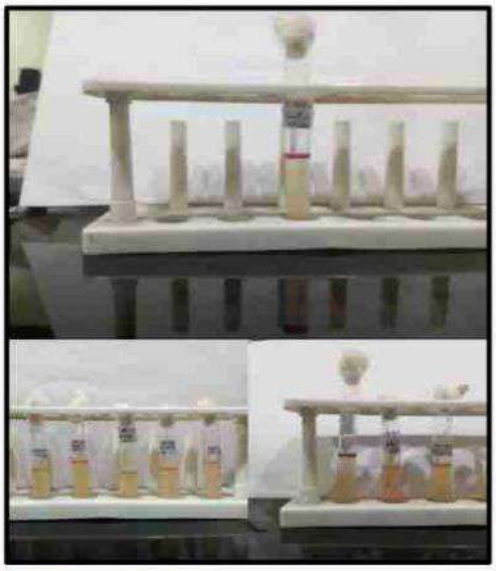


Figure 6 Indole tests

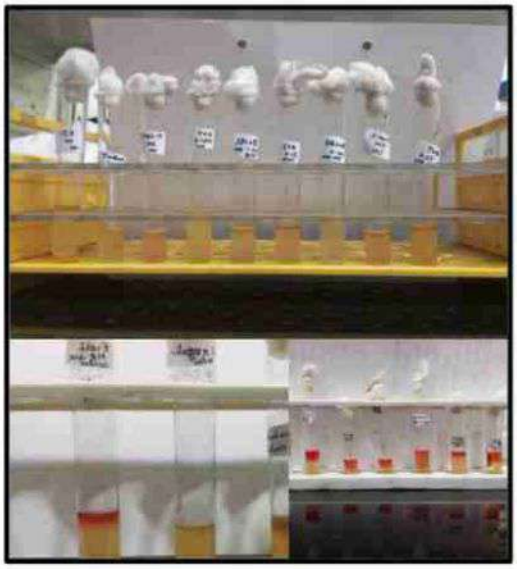


Figure 7:- MR TESTS

Figure 8 VP Tests

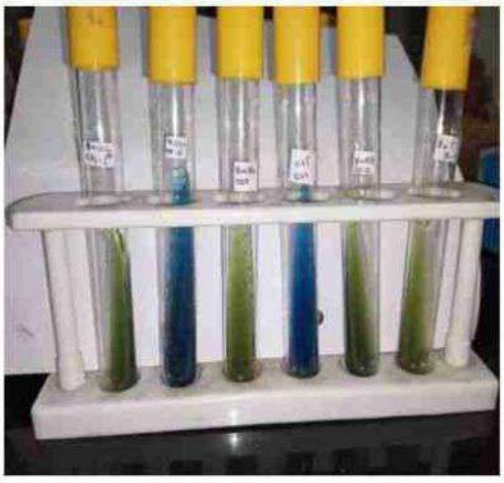
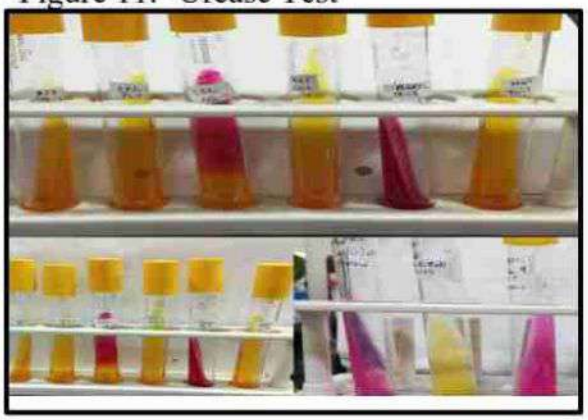


Figure 9 Citrate Tests

Observation Table for IMVi C

MAIN GROUP	MAIN SUBGROUP	MR 1,2	VP1,2,3	INDOLE	CITRATE1,2,3
CONTROL	IILEUM	positive	negative	positive	negative
AZIDE	COLON	positive	positive	negative	positive
AZIDE	COLON	positive	negative	negative	positive
AZIDE+BM	COLON 2	positive	negative	negative	positive
AZIDE +BM	COLON1	positive	negative	negative	positive
	COLON 2	positive	negative	negative	positive
AZIDE +BM	ILEUM	positive	negative	negative	positive
AZIDE+BM	COLON	positive	negative	negative	negative
BM	ILEUM	positive	negative	negative	negative
BM	ILEUM	positive	positive	negative	negative
Azide	Colon 2	positive	negative	negative	negative

Figure 11:- Urease Test



Urease Test

MAIN GROUP	• MAIN SUBGROUP	UREASE
CONTROL	• IILEUM	negative
AZIDE	• COLON	negative
AZIDE	• COLON	negative
AZIDE+ BM	• COLON 2	negative
AZIDE + BM	• COLON1	negative
AZIDE + BM	• COLON 2	negative
AZIDE +BM	• ILEUM	positive
AZIDE+BM	• COLON	positive
BM	• ILEUM	negative
BM	• ILEUM	positive
Azide	• Colon 2	negative

MAIN GROUP	MAIN GROUP	UREASE
AZIDE	COLON1	negative

AZIDE	COLON 3	positive
AZIDE	ILEUM 1	negative
AZIDE	ILEUM 2	positive
AZIDE	COLON 1	negative
AZIDE + BM	ILEUM 2	positive
AZIDE + BM	ILEUM 1	positive
AZIDE + BM	COLON 1	negative
AZIDE + BM	ILEUM 1	positive
BM	COLON 1	positive
BM	COLON 2	positive
BM	ILEUM 1	positive
BM	ILEUM 1	negative
BM	COLON 1	negative
BM	ILEUM 1	positive
BM	ILEUM 3	positive
BM	COLON 1	positive
BM	COLON 2	positive

TRIPLE SUGAR IRON TES

figure 11 TSI RESULTS

Observation Table :-

MAIN GROUP	MAIN SUBGROUP	TSI
CONTROL	IILEUM	negative
AZIDE	COLON	fermentation of glucose and lactose and sucrose
AZIDE	COLON	negative
AZIDE+ BM	COLON 2	negative
AZIDE + BM	COLON1	negative
AZIDE + BM	COLON 2	negative
AZIDE +BM	ILEUM	negative
AZIDE+BM	COLON	glucose fermentation only
BM	ILEUM	glucose fermented only
BM	ILEUM	glucose fermentation only
Azide	Colon 2	negative

MAIN GROUP	MAIN GROUP	TSI
AZIDE	COLON1	negative
AZIDE	COLON 3	positive
AZIDE	ILEUM 1	negative
AZIDE	ILEUM 2	negative
AZIDE	COLON 1	negative
AZIDE + BM	ILEUM 2	negative
AZIDE + BM	ILEUM 1	positive
AZIDE + BM	COLON 1	negative
AZIDE + BM	ILEUM 1	negative
BM	COLON 1	negative
BM	COLON 2	positive
BM	ILEUM 1	positive
BM	ILEUM 1	negative
BM	COLON 1	negative
BM	ILEUM 1	negative
BM	ILEUM 3	negative
BM	COLON 1	negative
BM	COLON 2	positive

ANTIBIOTIC SUSCEPTIBILITY TEST:-

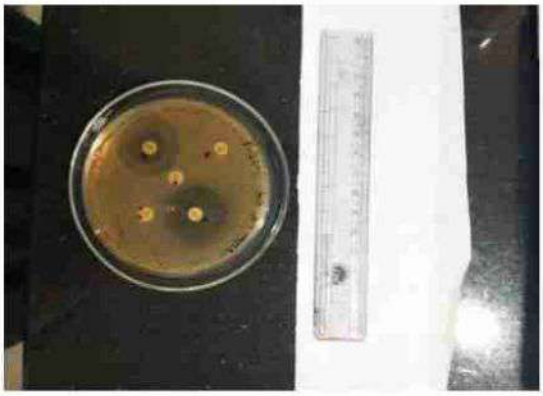


Figure12,13,14,15 Antibiotic Susceptibility tests

Observation Table for Antibiotic Susceptibility test

MAIN GROUP	MAIN SUBGROUP	CEPHALEXIN	AMPLICILIN	METHICILIN	VANCOMYCIN	TETRACY CLIN
CONTROL	IILEUM	1.25	1.45	1	No zone seen	No zone seen
AZIDE	COLON	1.35	1.5	1.5	No zone	0.3
AZIDE	COLON	1.2	1.5	No zone	No Zone	0.2
AZIDE+ BM	COLON 2	1	1.2	No zone	No zone	No zone
AZIDE + BM	COLON1	0.5	1.2	0.2	1.2	No zone
AZIDE + BM	COLON 2	1.6	1.4	1.2	No zone	1.8
AZIDE +BM	ILEUM	No zone	No zone	1.2	No zone	1.2
AZIDE+B M	COLON	1.5	No zone	1.2	No zone	1.2
BM	ILEUM	0.5	No zone	No zone	No zone	1.2
BM	ILEUM	0.2	No zone	.8	No zone	1.2
Azide	Colon 2	0.5	.2	.8	.6	.5

CATALASE and COAGULASE TEST RESULTS: -

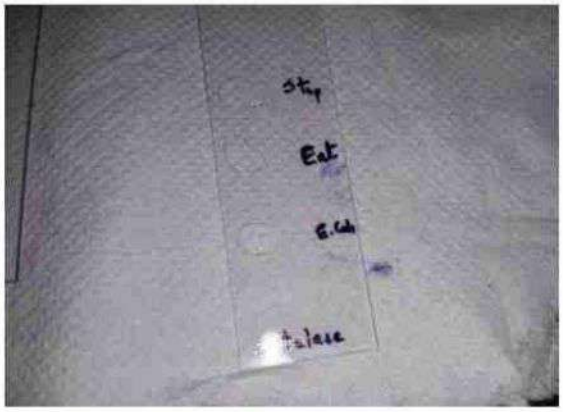


Figure 16:- Catalase control

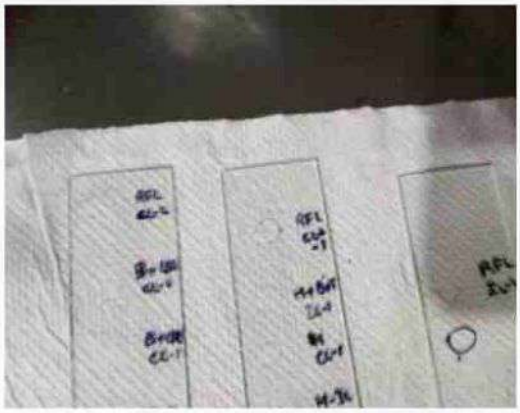


Figure 17:- Catalase Test

MAIN GROUP	MAIN SUBGROUP	Catalase	coagulase
CONTROL	IILEUM	Positive	negative
AZIDE	COLON	Positive	negative
AZIDE	COLON	Negative	positive
AZIDE+ BM	COLON 2	Negative	positive
AZIDE + BM	COLON1	Negative	positive
AZIDE + BM	COLON 2	Negative	positive
AZIDE +BM	ILEUM	Positive	negative
AZIDE+BM	COLON	Positive	negative
BM	ILEUM	Negative	positive
BM	ILEUM	Positive	negative
Azide	Colon 2	Positive	negative

MAIN GROUP	MAIN GROUP	Catalase	coagulase
AZIDE	COLON1	Positive	negative
AZIDE	COLON 3	Positive	negative
AZIDE	ILEUM 1	Negative	positive

AZIDE	ILEUM 2	Negative	positive
AZIDE	COLON 1	Positive	negative
AZIDE + BM	ILEUM 2	Negative	positive
AZIDE + BM	ILEUM 1	Positive	negative
AZIDE + BM	COLON 1	Negative	positive
AZIDE + BM	ILEUM 1	Negative	positive
BM	COLON 1	Positive	negative
BM	COLON 2	Negative	negative
BM	ILEUM 1	Negative	positive.
BM	ILEUM 1	Negative	positive
BM	COLON 1	Positive	negative
BM	ILEUM 1	Positive	negative
BM	ILEUM 3	Negative	positive
BM	COLON 1	Negative	positive
BM	COLON 2	Negative	positive

Figure 18 Coagulase Test

Figure 19 coagulase test

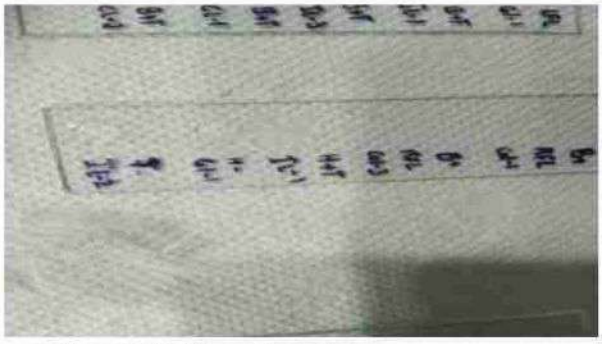
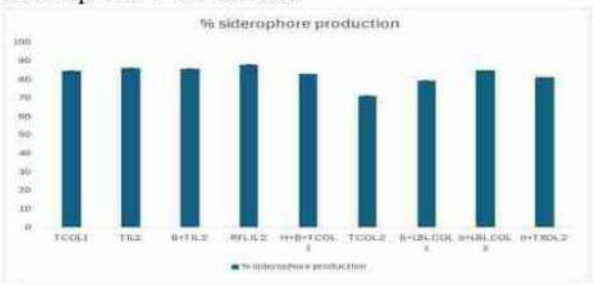
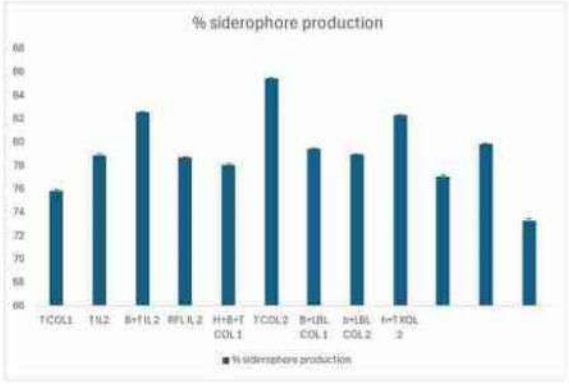
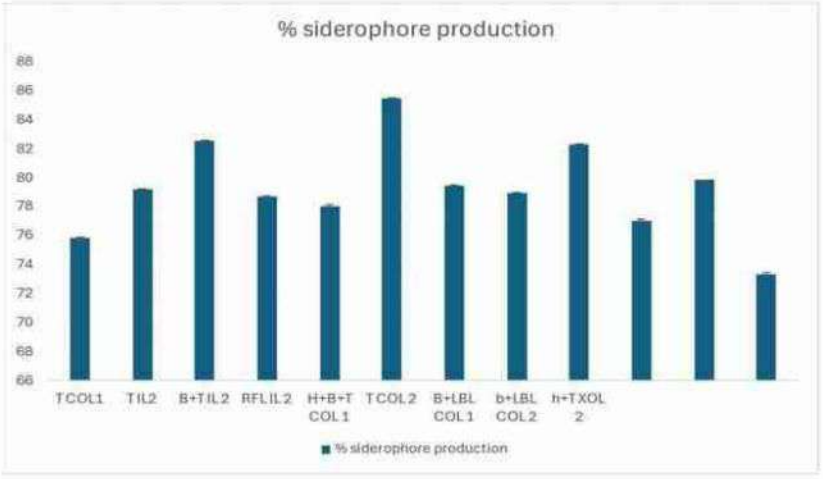


Figure 20:-Coagulase Test

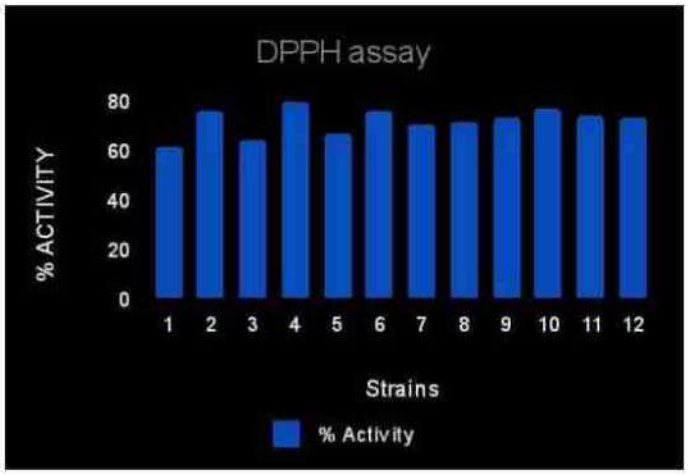
Siderophore Test Results:-







DPPH Results:-



Histology Images:-

Figure 21: -Ileum and colon images on 100 X

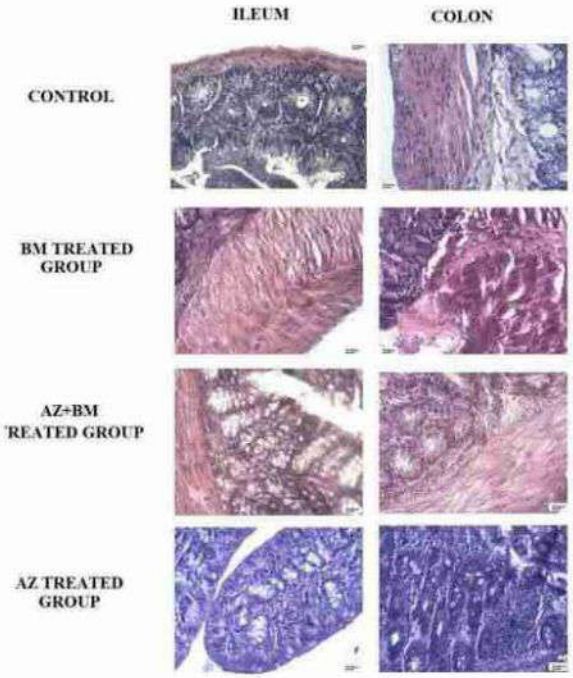
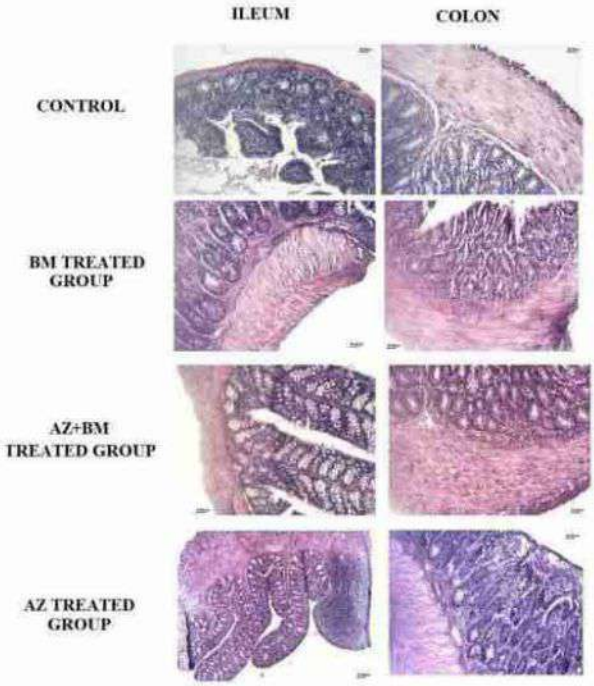


Figure 22: - Ileum and colon images(200x)



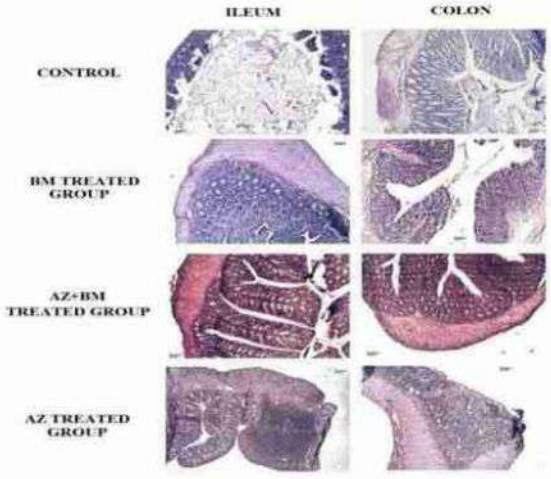


Figure 23 showing histology of ileum and colon in 400x

DISCUSSIONS:-

This study showed a mix of Gram-negative and Gram-positive bacteria, some from the gut of an Alzheimer's mice model. The bacteria behaved differently in basic biochemical tests—indole was mostly negative, while MR, VP, citrate, and urease results varied. This points to metabolic diversity, likely due to different genetic backgrounds or environmental conditions.

Antibiotic resistance was common across many strains, which is concerning and suggests possible exposure to antibiotics or gene sharing among bacteria.

Siderophore production also varied. Strains like BRL2 and HMT consistently produced more siderophores, which means they might be better at grabbing iron—a useful trait for plant growth. Others, like KOL2, produced less, possibly due to strain differences or testing conditions.

Overall, some strains show promise for use in agriculture or biotechnology, but antibiotic resistance highlights the need for careful screening.

CONCLUSION

This study looked at how *Bacopa monnieri* (Brahmi) affects gut microbes in mice with Alzheimer's-like symptoms (induced by sodium azide).
Mice treated with sodium azide showed memory loss, behavioral changes, and disturbed gut microbiota.
Brahmi treatment helped improve memory and behavior in mice.
It also showed a positive effect on gut bacteria—restoring balance and reducing harmful strains.
The combination of azide + Brahmi showed better results than azide alone.
These findings suggest that *Bacopa monnieri* might support brain health by acting through the gut-brain axis.
More research is needed, but Brahmi shows promise as a natural supplement for managing Alzheimer's symptoms.

FUTURE DIRECTIONS

Metabolite analysis of various parts of the guts and bacteria are linked to such metabolite generation can be the future direction.

Appendix

Figure 24 Gram staining

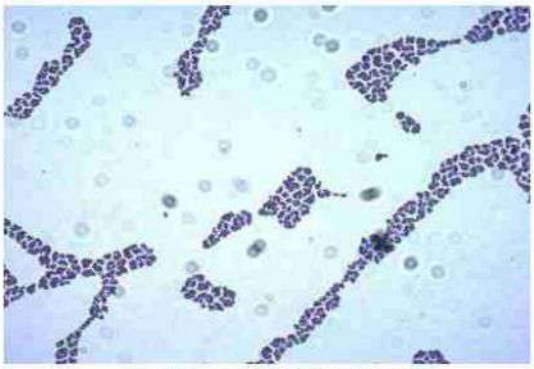


Figure 25 Gram Staining

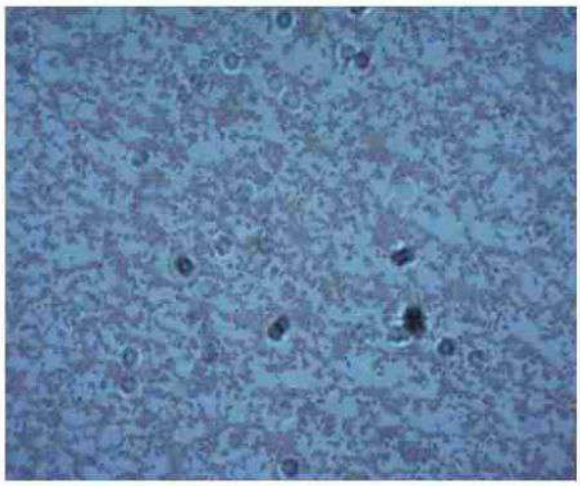


Figure 26-gram staining

figure 27 :- MR Tests

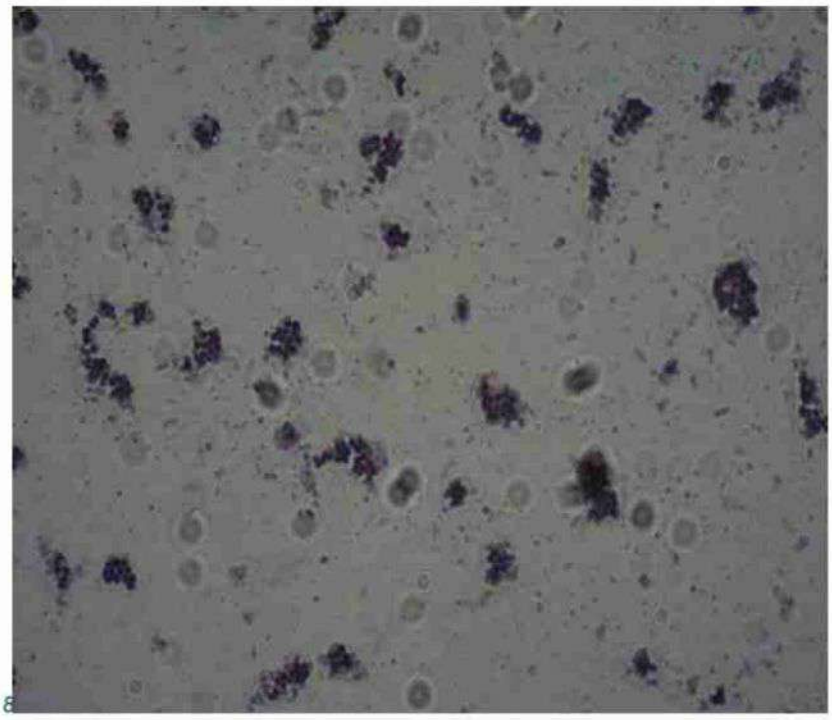


Figure 28

figure Urease

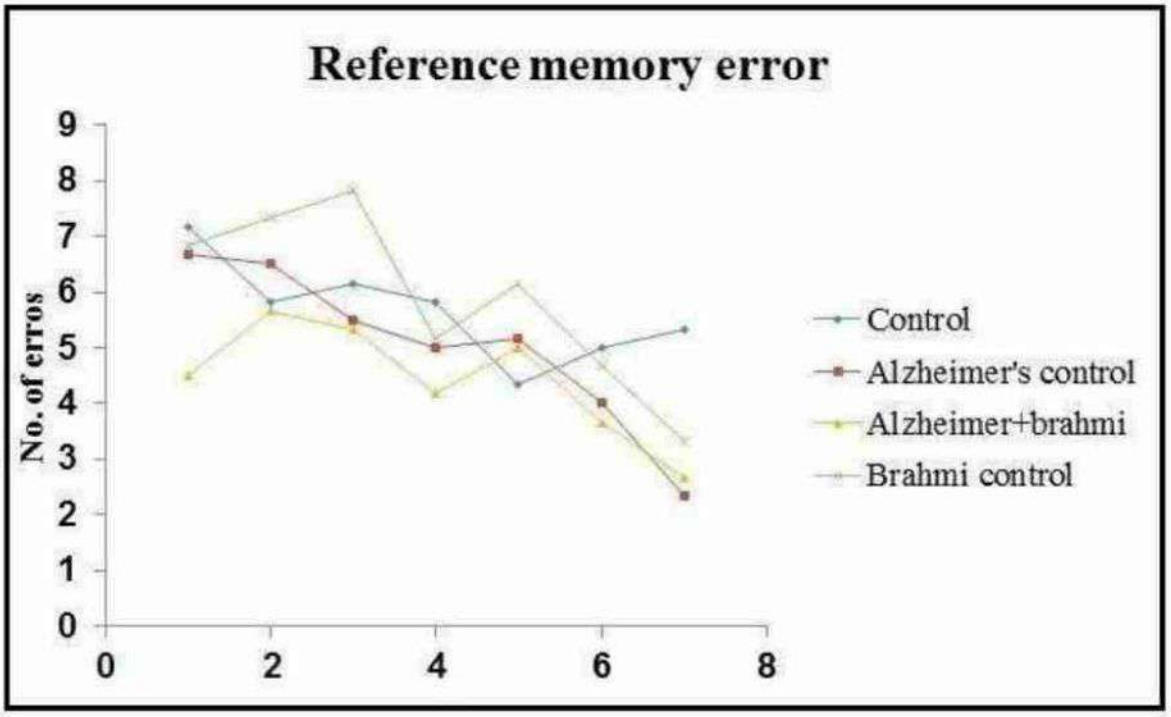


Figure 29 .1 Reference memory Error

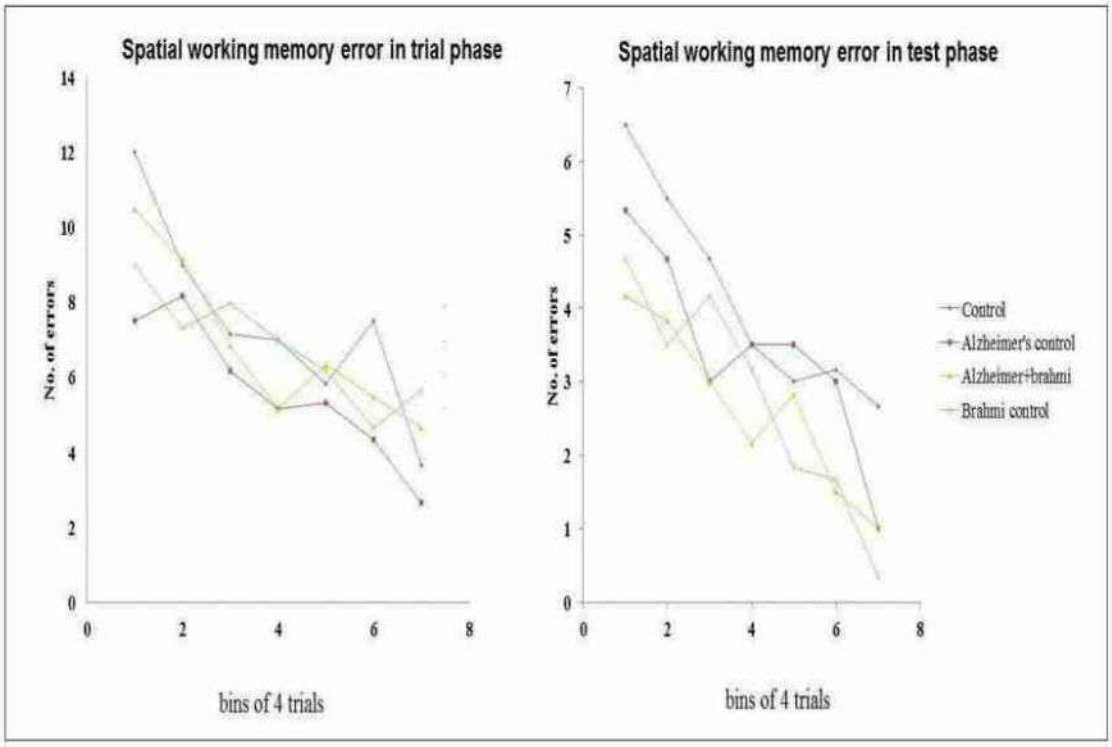


figure 29.2:- Spatial Working Errors

Figure 30

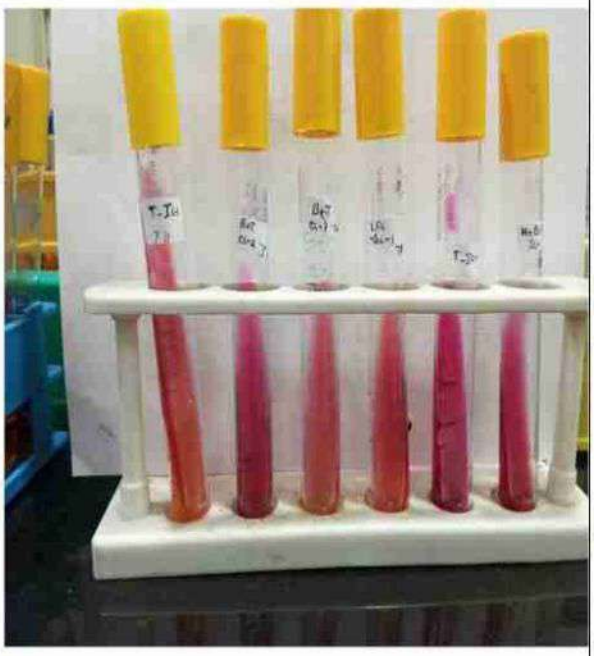


Figure 31 TSI

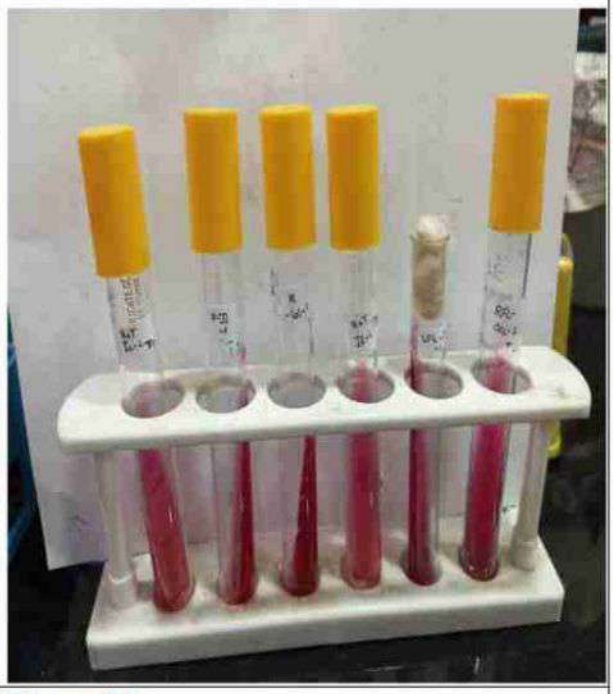


Figure 32

Figure 33

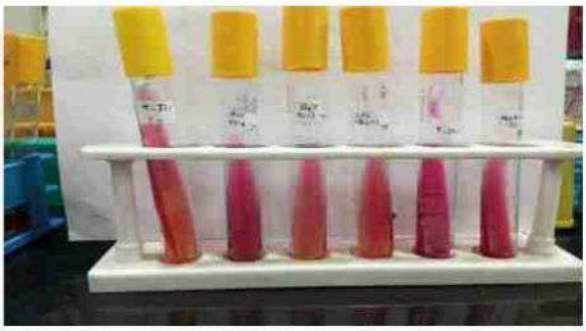


Figure 34 TSI

Figure 35 :-TSI TEST

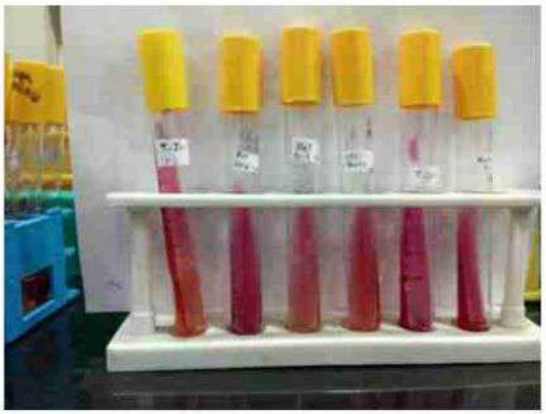


Figure 36 antibiotic susceptibility tests

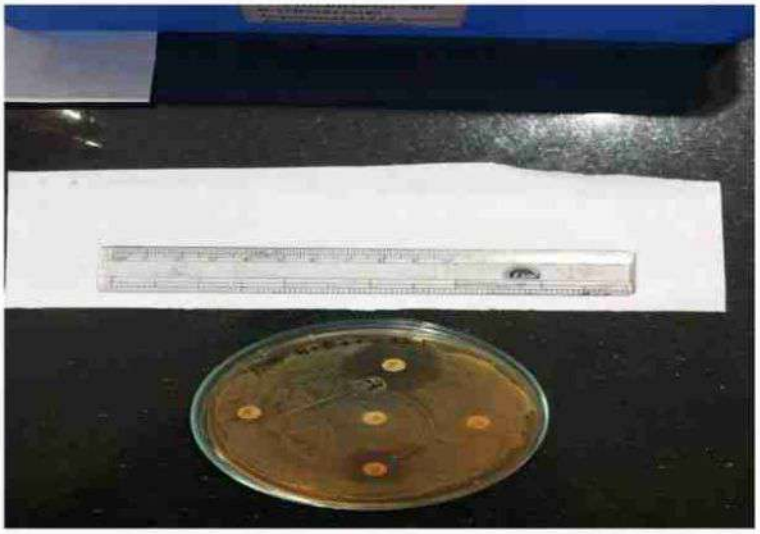


Figure 37 antibiotic susceptibility tests

Figure 38 Antibiotic Susceptibility Test

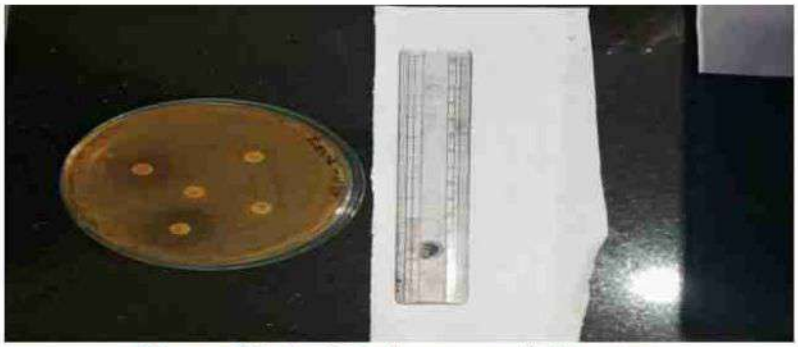


Figure 39 Antibiotic susceptibility test

Figure 40 Antibiotic Susceptibility test

Figure 41 Antibiotic Susceptibility test

Figure 42 Citrate Test

Figure 43 Citrate Test

Figure 44 CITRATE Test

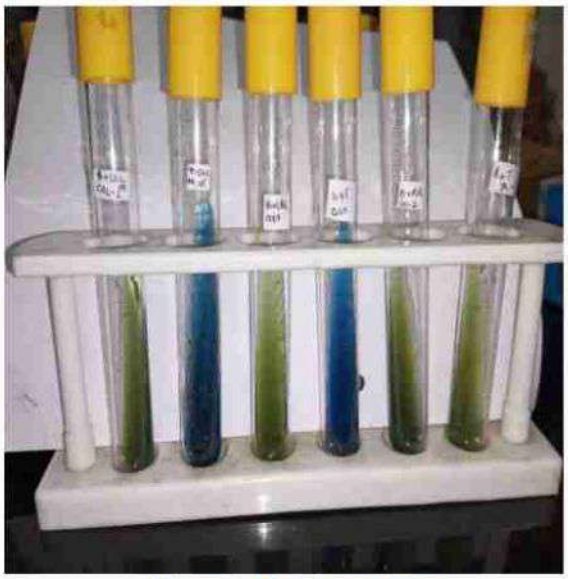


Figure 45 Citrate Test

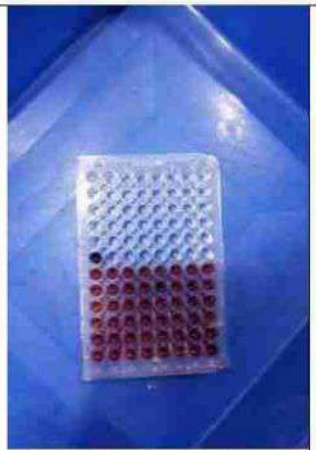


Figure 46 Siderophore

Figure 47 Siderophore Assay

Figure 48 Siderophore Assay

Figure 39 catalase

Figure 50catalase

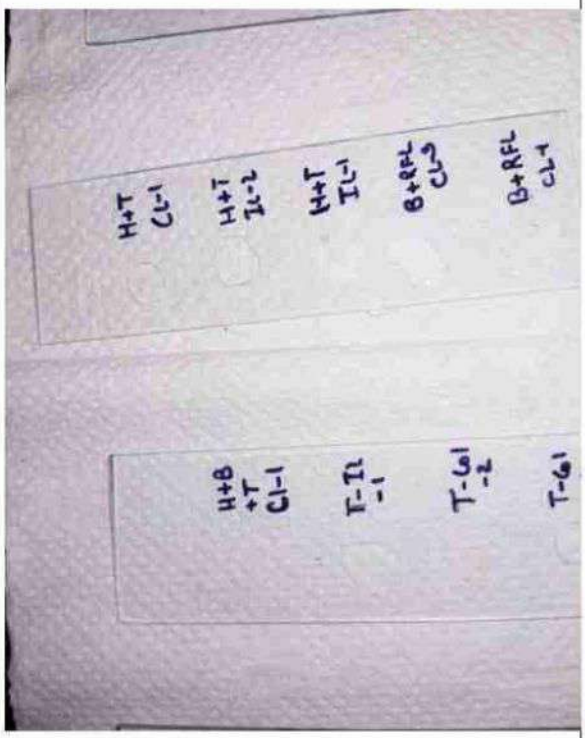


Figure 51catalase

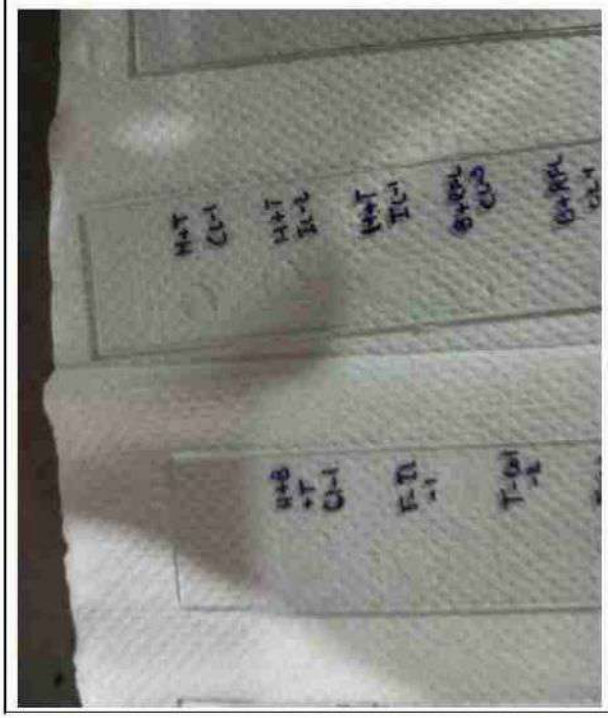
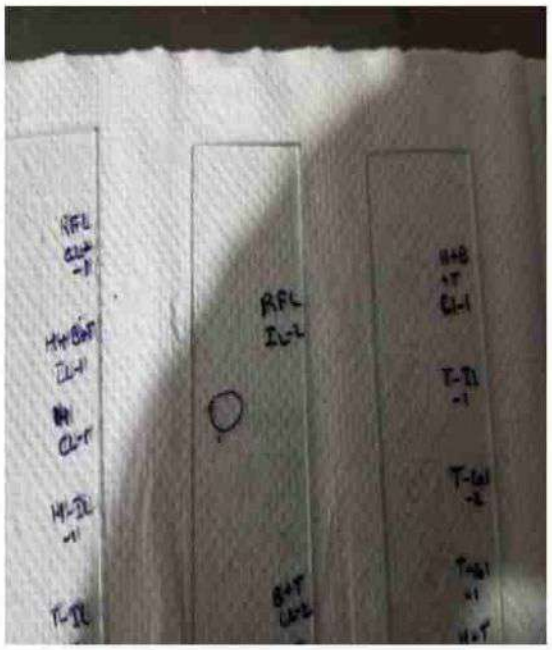


Figure 52 catalase



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